Plant Physiol. (1996) 112: 893–900

Rapid Communication

An Improved Green Fluorescent Protein Gene as a Vital Marker in Plants

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A synthetic green fluorescent protein (GFP) gene (*pgfp*) was constructed to improve GFP expression in plants. Corn and tobacco protoplast transient assays showed that *pgfp* gave about 20-fold brighter fluorescence than the wild-type gene (*gfp*). Replacement of the serine at position 65 with a threonine (*S65Tpgfp*) or a cysteine (*S65Cpgfp*) yielded 100- to 120-fold brighter fluorescence than wild-type *gfp* upon excitation with 490-nm light. Incorporation of a plant intron into the coding region yielded an additional 1.4-fold improvement, for a cumulative improvement of about 150-fold in fluorescence at 490-nm excitation. Various versions of *pgfp* were also stably introduced into corn, wheat, tobacco, and Arabidopsis plants. Bright-green fluorescence was observed with a fluorescence microscope in virtually all examined tissues of transgenic monocots and dicots. In the case of Arabidopsis, expression of the *pgfp* gene under the enhanced 35S promoter of the cauliflower mosaic virus produced green fluorescence that was readily detectable by eye using a hand-held, long-wave ultraviolet lamp and/or a black-light source.

Since the first demonstration of GFP from the jellyfish *Aequorea victoria* as a vital reporter for gene expression in both bacteria and *Caenorhabditis elegans* (Chalfie et al., 1994), GFP has attracted widespread interest and is considered to have several advantages over other visual marker genes. First, the fluorescence emission of GFP does not require a cofactor or a substrate; fluorescence results from an internal *p*-hydroxybenzylidene-imidazo-lidinone chromophore generated by cyclization and oxidation of a Ser-Tyr-Gly sequence at amino acid residues 65 to 67 (Cody et al., 1993). Detection of GFP in living cells thus only requires excitation by light at 395 or 470 nm. In contrast, the assay of GUS (Jefferson et al., 1987) expression is cytotoxic, firefly luciferase (Ow et al., 1986; Millar et al., 1995) requires an exogenous substrate (luciferin) for detection, and plant anthocyanins (Klein et al., 1989; Lloyd et al., 1992) are generally useful only in mature, differentiated cells.

The second advantage of GFP is that it is relatively small (26.9 kD) and can tolerate both N- and C-terminal protein fusions, lending itself to studies of protein localization and intracellular protein trafficking (Wang and Hazelrigg, 1994; Davis et al., 1995; Kaether and Gerdes, 1995). Another advantage of GFP is that GFP mutants with shifted wavelengths of absorption and emission have been isolated (Heim et al., 1994, 1995; Delagrave et al., 1995), which permits simultaneous use and detection of multiple reporter genes. In addition, some GFP mutants exhibit a more rapid formation of the chromophore and higher excitation peaks at 475 to 490 nm than does the wild-type GFP protein, which results in increased detection sensitivity (Heim et al., 1995).

Although GFP has tremendous potential as a vital marker for continuously monitoring gene expression in situ (Cubitt et al., 1995), its utility in plants remains to be determined. In spite of successful transient expression of the wild-type *gfp* in plant protoplasts (Hu and Cheng, 1995; Niedz et al., 1995; Sheen et al., 1995) and from virus-based vectors (Baulcombe et al., 1995; Casper and Holt, 1996), its expression in stably transformed plants has typically yielded very faint or no green fluorescence. It has been postulated that the poor expression of the wild-type *gfp* in plants was due to its high AT content and/or a cryptic intron sequence (Haseloff and Amos, 1995). Recently, a re-engineered *gfp* gene with the preferred codon usage of human proteins has been expressed at high levels in corn protoplasts and tobacco plants (Chiu et al., 1996). In this paper, we report that a synthetic *gfp* gene and its spectrally modified versions result in improved levels of green fluorescence in corn, wheat, tobacco, and Arabidopsis plants.

MATERIALS AND METHODS

Synthesis of *pgfp*

The synthetic *gfp* gene (*pgfp*, Fig. 1A) was designed using the method of Perlak et al. (1991) to encode a peptide sequence identical to the GFP protein of *Aequorea victoria* (Prasher et al., 1992). The *pgfp* gene was constructed by overlapping PCR and was ligated as a *Bam*HI-*Eco*RI frag-

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Abbreviations: CaMV35S, 35S RNA promoter of cauliflower mosaic virus; GFP, green fluorescent protein; *gfp*, gene encoding GFP; *gus*, GUS gene of *E. coli*; *nptII*, neomycin phosphotransferase II gene; *pgfp*, synthetic *gfp* gene with codon alterations for high-level expression in plants; *pgfp*-intron, the *pgfp* gene containing the potato ST-LS1 intron; *S65Cpgfp*, the same as *pgfp* except the Ser residue was mutated to Cys at amino acid position 65; *S65Tpgfp*, the same as *pgfp* except the Ser residue was mutated to Thr at amino acid position 65; *S65Tpgfp*-intron, the *S65Tpgfp* gene containing the ST-LS1 intron.

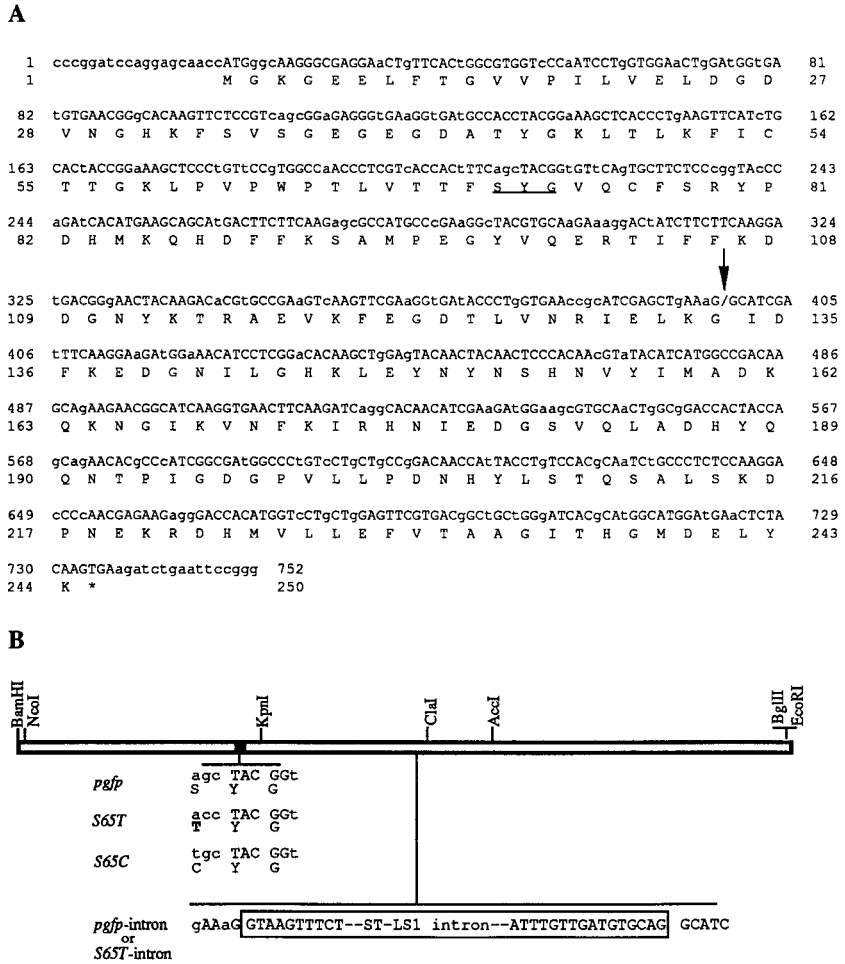

Figure 1. A, The *pgfp* nucleotide and amino acid sequences. The nucleotide differences compared with the native *A. victoria gfp* sequence are shown in lowercase letters. The chromophoric Ser-Tyr-Gly peptide sequence is underlined. The intron position is marked by a slash and an arrow. B, Diagram of various *gfp* versions. The chromophoric Ser-Tyr-Gly peptide sequence and the border sequences of the potato ST-LS1 intron are shown. *S65T*, *S65Tpgfp*; *S65C*, *S65Cpgfp*; *S65T*-intron, *S65Tpgfp*-intron.

ment into the same sites of a pBluescript II SK(−) (Stratagene) derivative that fused GFP to the N terminus of β-galactosidase and fluoresced in *Escherichia coli*. The sequence was confirmed by DNA sequencing (Sanger et al., 1977).

To make a wild-type *gfp* gene control with identical flanking untranslated regions, the wild-type *gfp* sequence was amplified by PCR from the plasmid pGFP (Clontech, Palo Alto, CA) with the oligonucleotide primers wtgfp-5′ (5′-CCCGGATCCAGGAGCAACCATGGGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCC) and wtgfp-3′ (5′ - CCCGGAATTCAGATCTTTATTTGTATAGTTCATCCATGCCATGTGTAATCCCAGCAGC), and ligated as a *Bam*HI-*Eco*RI fragment into the same sites of the same pBluescript II derivative. A fluorescent clone was sequenced and found to have only the intended changes in the untranslated regions and to be unchanged in the coding region.

Construction of *S65Tpgfp* and *S65Cpgfp*

A Ser-to-Thr (S65T) or Ser-to-Cys (S65C) mutation at amino acid position 65 (Fig. 1B) was generated by PCR-based mutagenesis of *pgfp* with the oligonucleotide primer *pgfp*-5′ (5′-CCCGGATCCAGGAGCAACCATGGGCAAGGGCGAGGAACTGTTCACTGGCGTGGTCCCAATCCTGGTGGAACTGGATGGTGATGTGAACGGGCACAA), corresponding to nucleotide positions 1 to 96 (Fig. 1A) and primers syn-S65T (5′-TGATCTGGGTACCGGGAGAAGCACTGAACACCGTAGGTGAAAGTGGTGACGAGGGTTGGC) or syn-S65C (5′-TGATCTGGGTACCGGGAGAAGCACTGAACACCGTAGCAGAAAGTGGTGACGAGGGTTGGC), corresponding to nucleotide positions 191 to 249 (Fig. 1A). Both S65T and S65C fragments were gel-purified, digested with *Bam*HI and *Kpn*I, and used to replace the corresponding region of similarly digested *pgfp*.

Construction of *pgfp*-Intron and *S65Tpgfp*-Intron

The second intron (IV2) of the potato ST-LS1 gene (Vancanneyt et al., 1990) was placed between nucleotide positions 398 and 399 of *pgfp* and *S65Tpgfp* (Fig. 1), the 5′ splicing site of the cryptic intron proposed by J. Haseloff and B. Amos (personal communication). This was achieved by PCR amplification of the ST-LS1 intron from the gus-intron chimeric gene (Vancanneyt et al., 1990) with intron 5′ primer (5′-GGTGAACCGCATCGAGCTGAAAGGTAAGTTTCTGCTTCTACCTTTGATAT) and intron 3′ primer (5′-CTTGAAATCGATACCTGCACATCAACAAATTTTG-

GTCATAT) and by overlapping PCR of the amplified intron and *Bam*HI-*Cla*I fragment of *pgfp* or *S65Tpgfp* with the intron 3′ primer and a *pgfp* primer (5′-GAACGGGCACAAGTTCTCCGTCAG), corresponding to nucleotide positions 85 to 108 (Fig. 1A). The amplified fragments were digested with *Kpn*I and *Cla*I and used to replace the corresponding regions of similarly digested *pgfp* and *S65Tpgfp* genes. The sequences of the resulting *pgfp*-intron and *S65Tpgfp*-intron gene were confirmed by the nucleotide sequence analysis.

Construction of Plant Expression and Transformation Vectors

The wild-type *gfp*, *pgfp*, *S65Tpgfp*, *S65Cpgfp*, *pgfp*-intron, and *S65Tpgfp*-intron were excised with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of the monocot expression vector pMON19470 (Fig. 2). In the resulting monocot expression vectors pMON30030 (wild-type *gfp*), pMON30029 (*pgfp*), pMON30037 (*S65Tpgfp*), pMON30038 (*S65Cpgfp*), pMON30048 (*pgfp*-intron), and pMON30049 (*S65Tpgfp*-intron), the *gfp* or *pgfp* genes were placed under the control of an enhanced CaMV35S promoter and located between the maize heat-shock protein 70 intron and the nopaline synthase polyadenylation region.

The *pgfp* and wild-type *gfp* genes in the pBluescript II SK(−) derivatives were excised with *Nco*I and *Eco*RI and cloned in the sense orientation into the same sites of the dicot expression vector pMON11740 (Fig. 2), resulting in pMON30058 (*pgfp*) and pMON30059 (wild-type *gfp*), respectively. In these dicot expression vectors (Fig. 2), the *gfp* genes were placed under the control of an enhanced CaMV35S promoter and located between the soybean heat-shock protein 17.9 leader and the nopaline synthase 3′ region. To construct dicot expression vectors for *S65Tpgfp*, *S65Cpgfp*, the *pgfp*-intron, and the *S65Tpgfp*-intron, the *Nco*I-*Cla*I fragments were excised from pMON30037, pMON30038, pMON30048, and pMON30049, and cloned into the same sites of pMON30058. The resulting constructs were designated pMON30060 for *S65Tpgfp*, pMON30061 for *S65Cpgfp*, pMON30062 for the *pgfp*-intron, and pMON30063 for the *S65Tpgfp*-intron (Fig. 2). For *Agrobacterium*-mediated transformation, the expression cassettes were excised from the respective expression vectors with *Not*I and cloned into the same site of the binary vector pMON18342 (Fig. 2) so that the direction of *gfp* or *pgfp* transcription was the same as that of NOS-*nptII*-NOS in pMON18342. The resulting binary vectors were designated pMON30064 for *pgfp*, pMON30065 for the wild-type *gfp*, pMON30066 for *S65Tpgfp*, pMON30067 for *S65Cpgfp*, pMON30068 for the *pgfp*-intron, and pMON30069 for the *S65Tpgfp*-intron (Fig. 2). The binary vectors were then transferred to *Agrobacterium tumefaciens* strain ABI by electroporation.

Transient Expression of Various Versions of *gfp* in Corn and Tobacco Protoplasts

Protoplast isolation and electroporation were performed using the methods of Sheen (1991) in corn leaves, and the

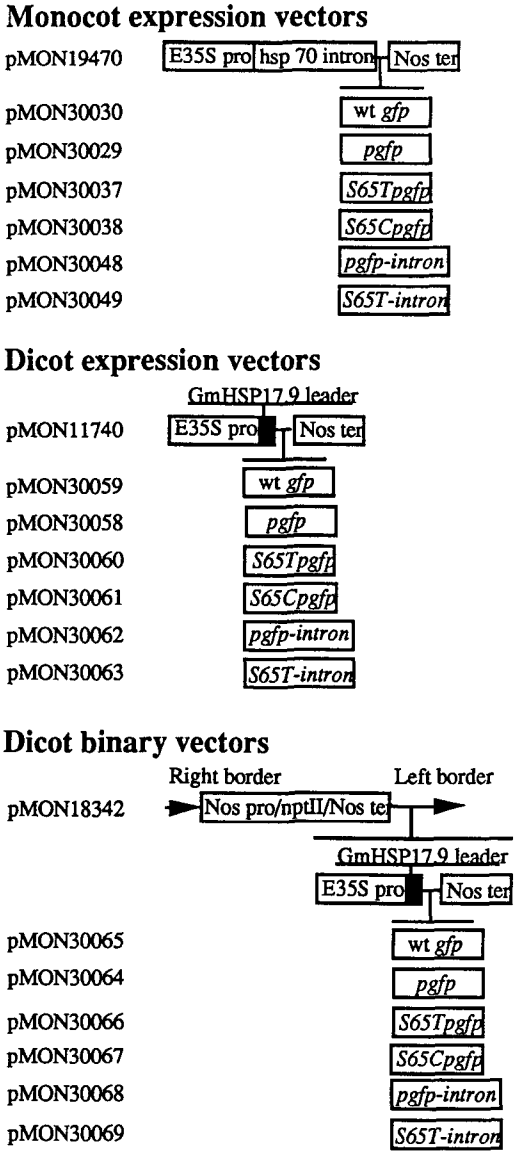

Figure 2. Linear structures of constructs used in this study. E35S pro, Enhanced CaMV35S promoter; hsp70 intron, the maize heat-shock protein 70 intron; Nos ter, nopaline synthase gene transcription terminator; Gm HSP17.9 leader, the soybean heat-shock protein 17.9 gene leader sequence; wt, wild type.

methods of Fromm et al. (1987) in tobacco cv Xanthi line D (TxD) suspension cells. Fifty micrograms of test vectors and 10 μg of internal control GUS vector pMON19648 were mixed and used for each electroporation. Electroporated cells were incubated for 2 d before harvest and assayed by 4-methylumbelliferyl β-D-glucuronide and GFP fluorometric assays. GFP fluorescence intensity was normalized by relative GUS activity for each sample.

Production of Transgenic Plants for the Expression of GFP

Transgenic corn plants were generated by co-bombardment of a type-II embryogenic callus with *gfp* transformation vectors, along with a kanamycin-selective

marker (pMON18369) vector (Fromm et al., 1990). Transgenic wheat plants were similarly produced using a biolistic transformation method (Vasil et al., 1993; Weeks et al., 1993). Transgenic Arabidopsis and tobacco were produced via *Agrobacterium* vacuum-infiltration transformation (Bechtold et al., 1993) and *Agrobacterium*-mediated transformation of leaf discs (Horsch et al., 1985), respectively.

4-Methylumbelliferyl β-D-Glucuronide Assay

GUS activity was determined by the method of Jefferson et al., (1987).

Detection of GFP by Fluorescence Spectroscopy

Protoplasts (3×10^6 to 4×10^6) were extracted in 300 μL of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, and 10 mM DTT (Chalfie et al., 1994). The cleared supernatants were analyzed with excitation at 395 and 490 nm in a luminescence spectrometer (model LS50, Perkin-Elmer).

Detection of GFP by Fluorescence Microscopy

Electroporated protoplasts and whole leaves of transgenic plants were examined using a fluorescence microscope (Photo 3, Zeiss) with a fluorescein isothiocyanate filter set (HQ480/40 excitation filter, HQ535/50 emission filter, Q505LP dichroic mirror; Chroma Technology, Brattleboro, VT). Photographs were taken using Kodak Ektachrome 160 film. No significant autofluorescence was seen using this filter combination.

Detection of GFP in Whole Plants

Plants were illuminated with a hand-held, long-wave UV lamp and/or a black-light source (General Electric BLB fluorescent) and photographed with 35-mm Kodak Ektachrome 400 film through a yellow-green filter.

RESULTS

Construction of Improved Versions of *gfp*

A synthetic plant *gfp* gene (*pgfp*) was synthesized by overlapping PCR and sequenced (Fig. 1A). The *pgfp* gene was expressed in *E. coli* as a fusion protein to β-galactosidase and produced fluorescent colonies when illuminated by a hand-held, long-wave UV lamp. The Ser residue at amino acid position 65 of wild-type GFP was mutated to Thr or Cys to produce the *S65Tpgfp* and *S65Cpgfp* mutants of *pgfp*, respectively (Fig. 1B). These mutants are excited at 490 nm (Heim et al., 1995), a preferred wavelength to avoid GFP photobleaching associated with excitation at 395 nm and a more useful wavelength for fluorescent microscopy (Cubitt et al., 1995). Additionally, the second intron (IV2) of the ST-LS1 gene (Vancanneyt et al., 1990) was introduced into the *pgfp* and *S65Tpgfp* coding sequences (Fig. 1B) to eliminate bacterial expression of the *pgfp* gene in *E. coli* (data not shown) and, presumably, in *Agrobacterium*, and to allow its use as a vital marker in monitoring early events in *Agrobacterium*-mediated plant transformation. In the resulting *pgfp*-intron and *S65Tpgfp*-intron genes, the ST-LS1 intron was positioned at the putative 5′ splicing site of the cryptic intron of wild-type *gfp* recognized in Arabidopsis (Haseloff and Amos, 1995) to produce an efficiently spliced intron.

Expression of Various Versions of *gfp* in Corn and Tobacco Protoplasts

The various versions of *gfp* were inserted into monocot and dicot expression vectors (Fig. 2), both of which utilize the enhanced CaMV35S promoter and the nopaline synthase 3′ region. The monocot expression vectors also include the hsp70 intron between the 35S promoter and the *gfp* coding regions. The dicot expression vectors contain the leader sequence of the soybean heat-shock protein 17.9 gene between the 35S promoter and the *gfp* coding regions. These *gfp* monocot and dicot expression vectors were electroporated into corn and tobacco protoplasts, respectively, and were allowed to express for 2 d before examination by fluorescence microscopy. Figure 3 shows corn protoplasts electroporated with the monocot vectors expressing either wild-type *gfp*, *pgfp*, or *S65Tpgfp* genes. Upon illumination with blue light (460–500 nm), protoplasts transformed with *S65Tpgfp*, *S65Cpgfp*, or the *S65Tpgfp*-intron displayed the brightest green fluorescence; protoplasts with *pgfp* or the *pgfp*-intron showed moderate fluorescence; and protoplasts with the wild-type *gfp* had the weakest fluorescence. The level of expression also correlated with the percentage of the protoplasts that showed fluorescence. Typically, green fluorescence was observed in virtually all viable proto-

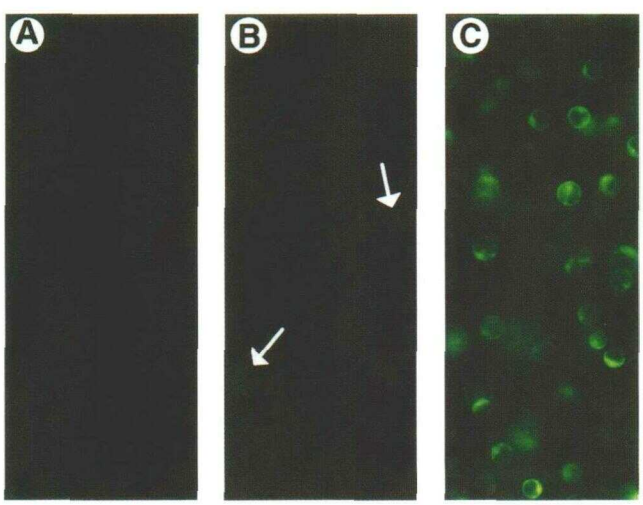

Figure 3. Comparison of green fluorescence intensity at 490 nm in corn protoplasts electroporated with pMON30030 containing wild-type *gfp* (A), pMON30029 containing *pgfp* (B), or pMON30037 containing *S65Tpgfp* (C). Electroporated protoplasts were examined using a fluorescence microscope (Photo 3, Zeiss) with a fluorescein isothiocyanate filter set. Photographs were taken using Kodak Ektachrome 160 film and the same camera settings were used for all three photographs.

plasts with *S65Tpgfp*, *S65Cpgfp*, or the *S65Tpgfp*-intron, most of the protoplasts with *pgfp* or the *pgfp*-intron, and less than 50% of the protoplasts with the wild-type *gfp*. In all cases the brightest fluorescence appeared to be associated with the cytoplasm; a large dark region, presumably the vacuole, was also observed (Fig. 3).

To quantitatively compare *gfp* expression levels, corn and tobacco protoplasts were electroporated with the various monocot and dicot *gfp* expression vectors, respectively, together with a *gus* construct (pMON19648) as an internal control. Extracts were prepared from the electroporated protoplasts after 2 d of incubation, and the supernatants were assayed for the level of GFP by fluorescence spectroscopy. The level of green fluorescence was normalized by the GUS internal control and is shown in Figure 4. Comparison of green fluorescence intensities produced by different versions of *gfp* and *pgfp* genes in the protoplasts was made after substraction of the background autofluorescence in the control protoplasts. In corn protoplasts *pgfp* typically produced 20- and 18-fold brighter fluorescence than the wild-type *gfp* with illumination at 395 and 490 nm, respectively. Similarly, *pgfp* produced 15- (395 nm) and 20- (490 nm) fold brighter fluorescence than the wild-type *gfp* in tobacco protoplasts. These data show that *pgfp* had a 15- to 20-fold improvement in green fluorescence compared with the wild-type *gfp* gene in both corn and tobacco protoplasts.

When excited at 490 nm, *S65Tpgfp* and *S65Cpgfp* produced a 5.1-fold brighter fluorescence than *pgfp*, and an 89-fold brighter fluorescence than the wild-type *gfp* in corn protoplasts. Likewise, tobacco protoplasts electroporated with *S65Tpgfp* and *S65Cpgfp* displayed fluorescence 5.8-fold brighter than *pgfp* and 117-fold brighter than the wild-type *gfp*. This additional 5.1- to 5.8-fold improvement of the *pgfp* mutants over *pgfp* correlated well with the 4- to 6-fold greater amplitudes of the excitation peaks at 490 nm of the *gfp* mutants than the wild-type *gfp* (Heim et al., 1995). Furthermore, as shown in Figure 4, insertion of the potato ST-LS1 intron into *pgfp* or *S65Tpgfp* resulted in 1.4-fold improvement of fluorescence over the same versions of *pgfp* lacking the ST-LSI intron in both corn (all monocot vectors still contain the hsp70 intron) and tobacco protoplasts. Thus, when illuminated at 490 nm, the overall improvements of the *S65Tpgfp*-intron over the wild-type *gfp* were 125- and 164-fold in corn and tobacco protoplasts, respectively.

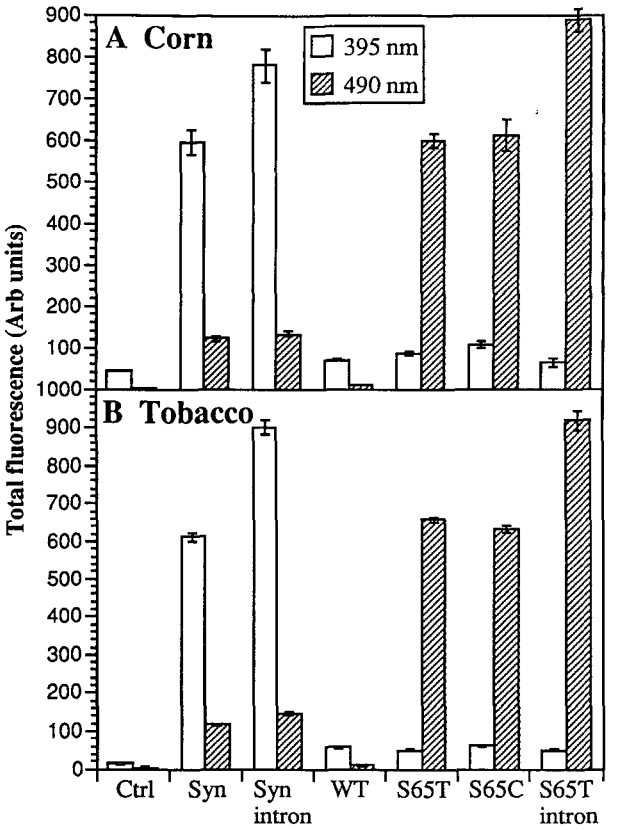

Figure 4. Quantitative green fluorescence analysis of various *gfp* versions in corn (A) and tobacco (B) protoplasts. Corn and tobacco protoplasts were isolated and electroporated as described in "Materials and Methods." GFP fluorescence at 510 nm of each sample was measured with excitation at 395 and 490 nm. The readings were normalized by the GUS activity of an internal control. Data presented in this figure are the average ± SE from at least four replicates before substraction of the background autofluorescence.

Expression of *pgfp* and Its Mutants in Transgenic Plants

Transgenic corn, wheat, Arabidopsis, and tobacco plants were produced to determine whether GFP can be used as a vital marker in monocot and dicot plants. The wild-type *gfp*, *pgfp*, *S65Tpgfp*, *S65Cpgfp*, *pgfp*-intron, and *S65Tpgfp*-intron constructs were all introduced into Arabidopsis by the vacuum infiltration method, and into tobacco by the *Agrobacterium*-mediated leaf disc transformation method. Only the *pgfp* and *S65Tpgfp* constructs were used to make transgenic corn and wheat plants by particle gun bombardment. Transgenic plants were examined under a fluorescence microscope. Both *pgfp* and its modified versions were expressed at high levels, leading to bright-green fluorescence in virtually all examined tissues of wheat, corn, tobacco, and Arabidopsis (Fig. 5). The proportion of highly fluorescent plants in the original transformants was quite high. For example, 9 out of 30 wheat plants transformed with *pgfp* and 9 out of 47 wheat plants transformed with *S65Tpgfp* showed bright-green fluorescence. Similarly, 4 out 16 corn plants transformed with *S65Tpgfp*, 31 out of 118 Arabidopsis plants transformed with the *S65Tpgfp*-intron, and 5 out of 36 tobacco plants with *pgfp* displayed strong green fluorescence.

Moreover, when Arabidopsis plants transformed with the *pgfp*-intron were illuminated with a hand-held, long-wave UV lamp and/or a black-light source, green fluorescence was readily detectable by eye in 20 out of 67 transgenic Arabidopsis plants (Fig. 6). In some cases whole plants fluoresced and in others green fluorescence was associated only with newly growing tissues. In contrast, no green fluorescence was observed in similar transgenic lines expressing the wild-type *gfp*. All of the results suggest that

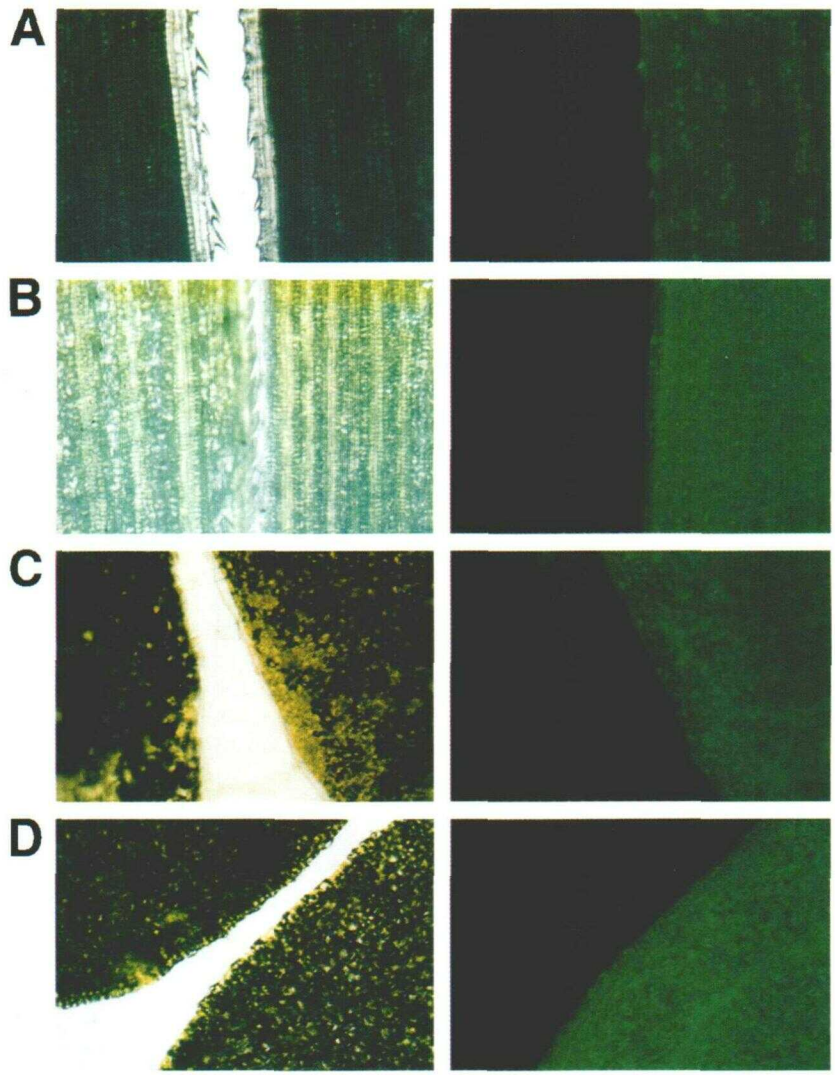

Figure 5. Detection of GFP in the leaves of transgenic plants. Leaves of wheat (A), corn (B), tobacco (C), and Arabidopsis (D) were illuminated with either visible light (left column) or blue light generated with a fluorescein isothiocyanate filter (right column). The leaf on the left in each photograph was nontransgenic, and the one on the right was transformed with *S65Tpgfp*.

pgfp and its modified versions were expressed at much higher levels in both monocots and dicots, resulting in strong green fluorescence in transgenic plants. The highly expressing plants grew well, developed normally, and set fertile seeds, suggesting that high levels of GFP are not toxic to plant cells.

DISCUSSION

A recent study found that a re-engineered *gfp* gene with the preferred codon usage of human proteins was highly expressed in corn protoplasts and transgenic tobacco plants (Chiu et al., 1996). We improved the expression of the native *gfp* gene in three steps. The first step involved modification of the *gfp* nucleotide sequence to increase its expression in plants, leading to about a 20-fold enhanced fluorescence in corn and tobacco protoplasts. This increase in fluorescence is consistent with the 20-fold increase in GFP protein levels observed in transient assays of similar synthetic *gfp* genes (Chiu et al., 1996). This single enhancement resulted in a green fluorescence in whole transgenic plants readily detectable by the naked eye with a hand-held, long-wave UV lamp. The second step was the incorporation of the S65T or S65C mutation (Heim et al., 1995) into our *pgfp*, resulting in another 5- to 6-fold improvement in green fluorescence when excited at 490 nm. The third step was the insertion of potato intron into *pgfp* or *S65Tpgfp*, resulting in another 1.3- to 1.5-fold improvement in green fluorescence. Overall, up to a 164-fold improvement of GFP expression was achieved from the *S65Tpgfp*-intron gene when illuminated at 490 nm. As a result, bright fluorescence was observed in all examined tissues of wheat, corn, tobacco, and Arabidopsis expressing these improved genes; there was little or no detectable GFP from the similar wild-type *gfp* gene transformants. These results demonstrate that *pgfp* and its modified versions express at much higher levels than the wild-type *gfp*, producing bright-green fluorescence in both transgenic monocots and dicots.

A key result of our work is that high levels of constitutively expressed GFP do not seem to interfere with the transformation, regeneration, or growth of plants. A prior study (Chiu et al., 1996) used an inducible promoter to

Figure 6. Detection of GFP in whole transgenic plants. Arabidopsis plants were illuminated with either visible light (left) or a hand-held, long-wave UV lamp and a black-light source (right). Arabidopsis plants in the pot at the left of each photograph were transformed with *pgfp*, and plants in the pot on the right were nontransgenic.

express *gfp*, possibly due to concerns about its effect on plant health if constitutively expressed. The ability to monitor constitutively highly expressed GFP in normal plants has potential uses such as identifying transformed cells or highly expressing cell lines nondestructively at very early stages; the labor required for subsequent subculture and evaluation of the transformed lines could be greatly reduced. They could also be useful in developing and optimizing transformation methods by continuously monitoring each transformation event at different stages. By using the *pgfp*-intron or the *S65Tpgfp*-intron constructs, early transformation events can be monitored in *Agrobacterium*-mediated plant transformation. These genes could also be useful in monitoring in vivo gene expression spatially and temporally at the subcellular, cellular, and plant levels, which have proven very difficult with existing markers such as GUS and firefly luciferase. Another use might be in mutant screens in which changes in gene expression are monitored by fluorescence screening. Additionally, since most GFP fusion proteins are still functional (Cubitt et al., 1995), *pgfp* and its mutants should increase the fluorescence signal when fused with cellular proteins to study protein localization and intracellular protein trafficking in living cells.

ACKNOWLEDGMENTS

We thank Dr. Curtis A. Holt for photographing whole fluorescent Arabidopsis plants and Nancy L. Mathis for transformation of tobacco.

Received June 27, 1996; accepted August 12, 1996.

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